

ACA CGA TTA ACA TCG CTA AG-3' (SEQ ID NO:24)) and a primer specific for downstream of C-terminus amino acid region of the same (T7Rpol-C: 5'-ATA TTT TAG CCA TGG TAT AGT GAG TCG TAT TGA TTT GGC G-3' (SEQ ID NO:25)) are synthesized. The phage DNA is used as a template for PCR, and thus an expression vector pT7R can be constructed (cf. Example 1). This expression vector can be transformed into *E. coli* DH5 α , and the transformed cells express a large amount of T7 RNA polymerase protein when isopropyl- β -Dthiogalactopyranoside (IPTG) is added.

In the substitute specification filed on May 20, 2002, please amend the paragraph beginning on page 22, line 12, as follows:

By using pT7R inserted with the wild type T7 RNA polymerase gene as a template, mutation was introduced by PCR into the region between the HpaI and NcoI restriction sites corresponding to the C-terminus side of the T7 RNA polymerase gene. More precisely, the region was divided into two fragments on the left side and right side of the nucleotide to be mutated, and these DNA fragments were amplified by PCR using primers F646Y(+) (5'-GTT GAC GGA AGC CGT ACT CTT TGG AC-3' (SEQ ID NO:26)) introduced with a mutation and F646Y(-) (5'-GTC CAA AGA GTA CGG CTT CCG TCA AC-3' (SEQ ID NO:27)), and primers T7RNAP-HpaI-N (5'-CGC GCG GTT AAC TTG CTT CCT AG-3' (SEQ ID NO:28)) and pTrc99a-PstI-C (5'-GCA TGC CTG CAG GTC GAC TCT AG-3' (SEQ ID NO: 29)), each containing a restriction cleavage site at the 5'-end. These DNA fragments had complementary regions, and denaturation, annealing and extension reactions of the regions were repeated to prepare a DNA fragment introduced

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F2 with the desired mutation. This DNA fragment was purified by collecting only a DNA fragment of a desired size through agarose gel electrophoresis, and this was re-amplified by using it as a template together with the primers T7RNAP-HpaI-N and pTrc99a-PstI-C, and cleaved with restriction endonuclease HpaI and PstI. This DNA fragment was separated by 1% agarose gel electrophoresis, and the band of the desired DNA fragment was cut out, and purified. The HpaI-PstI DNA fragment of pT7R was replaced with this DNA fragment to introduce a mutation. The resulting pT7R was transformed into *E. coli* DH5 α , and cells harboring the plasmid introduced with the mutation were selected. Finally, the nucleotide sequence was determined to confirm whether the mutation was introduced into the desired site. Thus, the expression plasmid pT7RF644Y for producing mutant T7 RNA polymerase F644Y was obtained. For the production of the mutant T7 RNA polymerase F644Y from this plasmid, expression could be induced by adding IPTG to the cultured *E. coli* cells harboring the plasmid, like the production of wild type T7 RNA polymerase.

In the substitute specification filed on May 20, 2002, please amend the paragraph beginning on page 23, line 16, as follows:

F3 First, a XhoI restriction site (CTCGAG) was introduced into the T7 RNA polymerase gene region of the expression vector pT7R having the wild type T7 RNA polymerase gene to facilitate the introduction of mutation. More specifically, the expression vector pT7R used as template was amplified by using a primer pair of primer ApaF1 (5'-CAT CTG GTC GCA TTG GGT CAC-3' (SEQ ID NO:30)) and primer Xho-R (5'-CCA AGT GTT CTC GAG TGG AGA-3' (SEQ ID NO:31)), and a primer pair of a

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primer Xho-F (5'-CTA AGT CTC CAC TCG AGA ACA CTT GG-3' (SEQ ID NO:32))

F3 and a primer AflII-R (5'-CAG CCA GCA GCT TAG CAG CAG-3' (SEQ ID NO:33)),

respectively. The former amplified DNA fragment was digested with restriction

endonucleases ApaI and XhoI, and the latter amplified DNA fragment with restriction

endonucleases AflII and XhoI, and they were ligated to the expression vector pT7R

preliminarily treated with ApaI and AflII by using T4 DNA ligase. This reaction product

was transformed into *E. coli* DH5 α , and several colonies grown on an agar plate

containing antibiotic ampicillin were obtained. Some of these colonies were selected and

cultured, and plasmid DNA was extracted from the cultured cells to obtain plasmid pT7R-

Xho in which a XhoI restriction site was introduced in the T7 RNA polymerase gene region

(see Figure 10). Presence of this XhoI site can be confirmed by cleavage by a treatment

with the restriction endonuclease XhoI, and nucleotide sequencing of the DNA. Using this

plasmid pT7R-Xho as a template, PCR was performed with a primer pair of primer Xho-R

and primer 667R (5'-GCT GAG TGT ACA TCG GAC CCT-3' (SEQ ID NO:34)), and a

primer pair of a primer 667F (5'- of -GCT GAG TGT ACA TCG GAC CCT-3' (SEQ ID

NO:35)) and a primer AflIIR. The PCR products were directly used as templates for the

nucleotide sequencing of the DNA to determine the sequences of the primers 667R and

667F. Then, they were subjected to electrophoresis on 2% agarose gel (Agarose X from

Nippon Gene was used as the agarose) respectively, and bands corresponding to DNA

fragments of the desired sizes were cut out to purify the DNA fragments by using Gene

Pure Kit. The purified two kinds of DNA fragments were mixed, and used as templates for

PCR using the primers XhoF and AflIIR. After confirming that the amplified DNA

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fragment was the desired fragment by restriction mapping and DNA sequencing, the fragment was digested with restriction endonucleases XhoI and AflII, and the resulting fragment was ligated to the plasmid pT7R-Xho preliminarily treated with restriction endonucleases XhoI and AflII by using T4 DNA ligase. This reaction product was transformed into *E. coli* DH5 α , and several colonies of the cells grown on an agar plate containing antibiotic ampicillin were obtained. Some of these colonies were selected and cultured, and plasmid DNA was extracted from the cultured cells. The plasmid DNA was confirmed if it was introduced with the desired mutation by DNA sequencing to finally construct an expression plasmid pT7RL665P/F667Y for producing the mutant T7 RNA polymerase L665P/F667Y (see Figure 12) . For the production of the mutant T7 RNA polymerase L665P/F667Y from this plasmid, expression could be induced by adding IPTG to the cultured *E. coli* cells harboring the plasmid, like the production of wild type T7 RNA polymerase.

In the substitute specification filed on May 20, 2002, please amend the paragraph beginning on page 32, line 18, as follows:

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PCR was performed by using the expression plasmid producing the mutant T7 RNA polymerase L665P/F667Y as template together with a primer pair of the primer Xho-F and the primer T7-DOUBLE-R (21-mer: 5'-CTCTTGACCCGTAAGCCAG-3' (SEQ ID NO:36)) or a primer pair of the primer T7-DOUBLE-F (29-mer: 5'--TTACGGGTCAAAGAGTACGGCTTCCGTC-3' (SEQ ID NO:37)) and the primer AflII-R. The PCR products were directly used as templates and determined for DNA sequences

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to confirm the sequences of the primers T7-DOUBLE-R and T7-DOUBLE-F. Each of the products was subjected to electrophoresis on 2% agarose gel to purify DNA fragment of the intended size. The purified two kinds of DNA fragments were mixed, and used as template for PCR using the primers XhoF and AflIIR. After confirming that the amplified DNA fragment was the desired fragments by restriction mapping and DNA sequencing, the fragment was digested with restriction endonucleases XhoI and AflII, and the resulting fragment was ligated to the plasmid pT7RL665P/F667Y preliminarily treated with restriction endonucleases XhoI and AflII by using T4 DNAligase. This reaction product was transformed into *E. coli* DH5 α , and several colonies of the cells grown on an agar plate containing antibiotic ampicillin were obtained. Some of these colonies were selected and cultured, and plasmid DNA was extracted from the cultured cells. The nucleotide sequence of the plasmid DNA was sequenced to confirm that the desired mutation was introduced, and thus an expression plasmid pT7RF644Y/L665P/F667Y for producing the mutant T7 RNA polymerase F644Y/L665P/F667Y was finally constructed (see Figure 17). For the production of the mutant T7 RNA polymerase F644Y/L665P/F667Y from this plasmid, expression could be induced by adding IPTG to cultured *E. coli* cells harboring the plasmid, like the production of the wild type T7 RNA polymerase.

In the substitute specification filed on May 20, 2002, please amend the paragraph beginning on page 35, line 10 (6th line following Table 1), as follows:

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As the template for PCR, human thyroid-stimulating hormone (hTSH- β) cDNA subcloned into a plasmid derived from BS750 having T7 promoter was used. By using this